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Subacute and Subchronic Toxicity Studies of Palm Vitamin E in Mice

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ABSTRACT

Palm oil is a rich source of vitamin E, especially the tocotrienols. It had been shown in previous studies to be effective in preventing and treating experimentally induced osteoporosis in laboratory rats. The objective of this study was to determine the subacute and subchronic toxic effects of palm vitamin E extract on mice. This was part of an ongoing effort to determine the potential for use of palm vitamin E as an anti-osteoporotic agent. The doses used in this study were 200, 500 and 1000 mg kg^{-1}. Treatment period was 14 days for the Subacute Toxicity Study and 42 days for the Subchronic Toxicity Study. The parameters measured were Bleeding Time, Clotting Time, serum aspartate aminotransferase, serum creatinine as well as liver and kidney weights. The results showed that the Bleeding and Clotting Times were significantly prolonged in the 500 and 1000 mg kg^{-1} groups in both the Subacute and Subchronic Toxicity Studies. Serum creatinine was raised in the 500 and 1000 mg kg^{-1} group for the Subchronic Toxicity Study. Kidney weights were increased in the 200 and 500 mg kg^{-1} groups for the Subacute Toxicity Study and in the 1000 mg kg^{-1} group for the Subchronic Toxicity Study. No changes in serum aspartate aminotransferase levels or in liver weights were seen in both the Subacute and Subchronic Toxicity Studies. In conclusion, large doses of palm vitamin E in animals well above the effective dose used to prevent and treat osteoporosis may cause bleeding tendency and renal impairment but there was no liver toxicity.

Key words: Subacute, subchronic, toxicity studies, palm vitamin E, mice

INTRODUCTION

Palm oil is a rich source of vitamin E. Vitamin E in palm oil consists of 28% alpha-tocopherol, 29% alpha-tocotrienol, 28% gamma-tocotrienol and 14% delta tocotrienol (Sundram *et al*., 2003). Many studies have been done on the effects of vitamin E in various disease conditions, such as cardiovascular disease (Meydani, 2000; Visioli and Hagen, 2007), neurodegenerative disease (Ricciarelli *et al*., 2007), cancer (Fu *et al*., 2009), experimentally-induced gastritis (Mtgapor *et al*., 2006; Nur Azlina *et al*., 2005) and other diseases. The effects of vitamin E on immune and inflammatory responses had also been studied (Wu and Meydani, 2008). Some of the studies above showed beneficial effects, some did not show any distinct advantage, while some indicated that vitamin E may actually be detrimental. However,most of the studies quoted used synthetic alpha-tocopherol as the vitamin E, except for Mtgapor *et al*. (2006) and Nur Azlina *et al*. (2005) which used palm tocotrienols. Our own animal studies found that tocotrienol-rich vitamin E mixture extracted from palm oil was effective in prevention and treatment of osteoporosis due to various

causes (Hermizi *et al.*, 2009; Nazrun *et al.*, 2005, 2008; Ima-Nirwana and Sunahiza, 2004). In fact, palm vitamin E extract was also able to improve bone structure in normal male rats (Shuid *et al.*, 2010). Our previous studies found that tocotrienols were more effective than alpha-tocopherol in prevention and treatment of experimental osteoporosis (Ahmad *et al.*, 2005). In all our studies, the effective dose of the palm vitamin E extract used was 60 mg kg^{-1}.

Other studies have found that tocotrienols were more effective proapoptotic agents than tocopherols, thus better anticancer agents (Constantinou *et al.*, 2008). Tocotrienols are shown to possess the ability to combat cancer *in vitro* and *in vivo*, evidently independent of their antioxidant activity. The discovery of the antiangiogenic, antiproliferative and apoptotic effects of tocotrienol, as well as its role as an inducer of immunological functions not only reveals a new horizon to approach tocotrienol as a potent antitumor agent, but also reinforces the notion that tocotrienols are indeed more than just antioxidants (Nesaretnam, 2008).

However, several reports were published showing that supplementation with vitamin E was associated with increased mortality and an increased risk of gastrointestinal cancer and heart failure (Eidelman *et al.*, 2004; Bjelakovic *et al.*, 2004; Miller *et al.*, 2005; Lonn *et al.*, 2005). Vitamin E did not provide benefit in mortality compared with control treatment or significantly decrease risk of cardiovascular death or cerebrovascular accident (Vievkananthan *et al.*, 2003). As with the previous studies from other researchers, most of these studies were done on alpha-tocopherol. Yu *et al.* (2006) studied increasing doses of tocotrienol-rich fraction of palm oil on 5-week old female chicks and found no significant changes in weight gain, weight of organs and feed consumption, suggesting that high levels of tocotrienol-rich fraction and its constituent tocols are free of side effects. They further suggested that the safe dose of various tocotrienols for human consumption is 200-1000 mg day^{-1}. However, a review article by Garewal and Diplock (1995) mentioned that in patients with vitamin K deficiency or on anticoagulant drugs such as warfarin, high doses of alpha-tocopherol may increase the bleeding tendency. Marsh and Coombes (2009) found that alpha-tocopheryl succinate plus lipoic acid supplementation in male rats increased bleeding tendency via an intrinsic coagulation pathway and even suggested that such supplementation may benefit patients with cardiovascular disease who exhibit elevated levels of coagulation and oxidative stress. However, there are no reports to date of the effects of tocotrienols on coagulation and bleeding. Other researchers did not find any acute or subacute toxic effects using doses up to 3% tocotrienol in diet of rats (Nakamura *et al.*, 2001).

In this experiment, subacute and subchronic toxic studies were done on laboratory mice given several doses of palm vitamin E extract. Biochemical and haematological parameters were measured to determine whether large doses of palm vitamin E extract pose any potential serious adverse effects. Approximately 20% of the vitamin E content in the extract was made up of α-tocopherol, while the rest were tocotrienol isomers of α, γ and δ. The reason a mixture was used in this study and in most of our previous studies is that it is more commercially viable to produce a mixture than to isolate a pure isomer from a naturally occuring plant oil.

MATERIALS AND METHODS

This research was done in the Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia between June 2007 to May 2008.

Animals: A total of 64 female mice aged 1-2 months, weighing 20-30 g were used, 32 for the Subacute Toxicity studies and 32 for the Subchronic Toxicity studies.

Palm tocotrienols: Palm vitamin E extract (PVE) was a gift from the Malaysian Palm Oil Board (MPOB). It contained 18.43% α-tocopherol, 14.62% α-tocotrienol, 32.45% γ-tocotrienol and 23.93% δ-tocotrienol. The doses used were 200, 500 and 1000 mg kg^{-1}. The PTT was given by oral gavage. The control group was given vehicle vitamin E-free palm oil.

Subacute toxicity study: Thirty-two animals were divide into 4 groups of 8 animals each and given the following treatment: (1) Vehicle; (2) PVE 200 mg kg^{-1}; (3) PVE 500 mg kg^{-1} and (4) PVE 1000 mg kg^{-1}. Daily doses of the PVE were given for 14 days. Renal function, liver function, bleeding and clotting times were determined before and after the treatment period.

Subchronic toxicity study: Another group of thirty-two animals were treated as above for 42 days. The same parameters were measured before and after the treatment period.

Parameters: Body weight and the wet weights of the liver and kidneys were measured using a digital balance (FX300, Japan). Bleeding time was determined by cutting off the tip of the tail about 0.5 cm from the end. The time taken for the bleeding to stop was recorded as the Bleeding Time. A drop of blood was placed on a slide. A needle was used to gently stir the drop of blood. The time taken for the first fibrin thread to develop was taken as the Clotting Time.

Renal function and liver function were determined by measuring serum creatinine and aspartate aminotransferase respectively using the Selectra E autoanalyser (Vital Scientific, Netherlands).

The Toxicity Studies were conducted according to the OECD Guidelines for Testing of Chemicals, and were approved by the UKM Animal Ethics Committee: FAR/2007/IMA/10-JULY/195-JULY-2007-MAY-2008.

Statistical analyses: Data obtained were analysed by using Analysis of Variance (ANOVA) by means of Statistical Package for Social Sciences (SPSS) version 13. All the data was tested using the Kolmogrov-Smirnov test and found to be normally distributed. The paired t-test was used for comparison within groups, and the one-way ANOVA followed by Tukey's post-hoc test was used for comparison between groups. The results were presented as Mean±SEM.

RESULTS

Mortality: No deaths were reported in the Subacute Toxicity Study. However, in the Subchronic Toxicity Studies, 1 death was recorded in the 200 mg kg^{-1} group. 3 deaths in the 500 mg kg^{-1} group and 1 death in the 1000 mg kg^{-1} group. The first death occurred in the fourth week of treatment in the 1000 mg kg^{-1} group.

Body weight: No significant differences were noted in weight gain between all groups in the Subacute Toxicity Study (Fig. 1). However, body weight was lower in the 500 mg kg^{-1} group from week 5 onwards; and for the 1000 mg kg^{-1} group from week 4 onwards compared to all the other groups for the Subchronic Toxicity Study (Fig. 2).

Bleeding and clotting times: Bleeding time was increased in the 500 mg kg^{-1} group in the Subacute Toxicity Study (Table 1) and 500 and 1000 mg kg^{-1} groups in the Subchronic

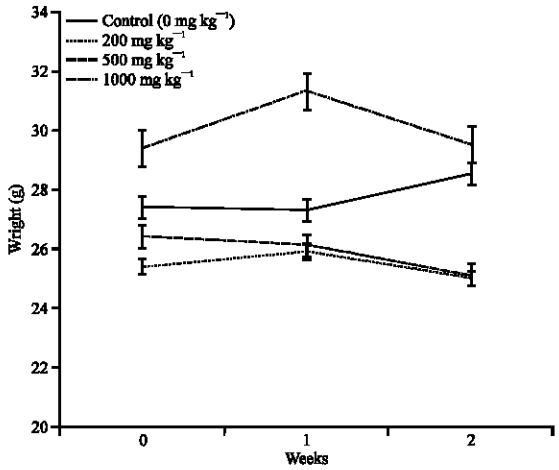

Fig. 1: Body weights of mice in the subacute toxicity study. No significant difference before and after treatment was found in all the groups. No significant difference between groups was seen both before and after treatment was started

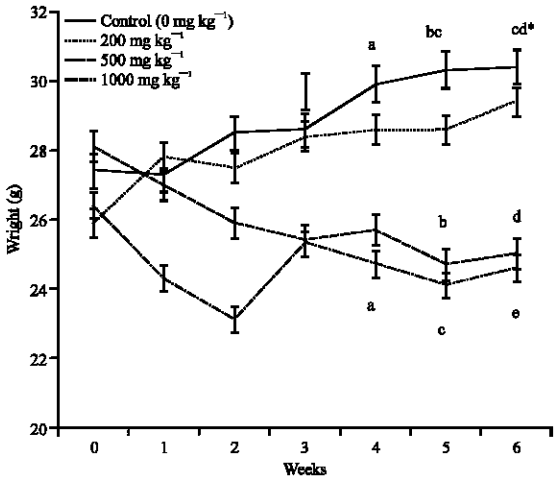

Fig. 2: Body weights of mice in the subchronic toxicity study. The symbol * indicates significant difference before and after treatment within each group at $p<0.05$. The same alphabet indicates significant difference between groups at $p<0.05$

Table 1: Subacute toxicity studies

PTT dose	Bleeding time (min)		Clotting time (min)		AST (U L^{-1})		Creatinine (umol L^{-1})	
(mg kg^{-1})	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
0	4.9±0.9	4.5±0.7	2.3±0.3	1.9±0.2$^{a\,b}$	148.4±16.1	126.8±20.7	37.4±1.0	41.1±0.6
200	8.8±1.4	10.4±1.7	3.5±0.2	2.4±0.2	255.5±57.7	177.4±21.4	35.1±1.9	37.4±1.7
500	6.4±0.7	10.2±1.5*	3.1±0.3	3.7±0.2^{a}	190.8±42.9	136.8±7.8	36.3±1.9	36.8±1.7
1000	7.9±1.5	8.7±3.0	2.8±0.3	3.2±0.3^{b}	184.3±2.2	194.9±37.4	38.2±2.2	42.6±2.4

The symbol *indicates significant difference before and after treatment within each group at $p<0.05$. The same alphabet indicates significant difference between groups at $p<0.05$

Toxicity Study (Table 2). Clotting time was increased in the 500 and 1000 mg kg^{-1} groups in both the Subacute and Subchronic Toxicity Studies (Table 1, 2).

Table 2: Subchronic toxicity studies

PTT dose	Bleeding time (min)		Clotting time (min)		AST (U L^{-1})		Creatinine (umol L^{-1})	
(mg kg^{-1})	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
0	4.9±0.9	4.5±0.8^{ab}	2.3±0.3	1.9±0.2^{ab}	148.4±16.1	126.8±20.7	37.4±1.0	41.1±0.6*^{ab}
200	6.7±0.8	6.0±0.9	2.5±0.3	4.1±0.7	224.4±28.9	172.1±29.7	37.3±1.3	45.8±1.1*
500	7.9±1.2	10.4±1.7^{a}	3.1±0.3	5.1±1.0^{a}	238.4±45.1	216.6±41.4	39.3±0.7	54.2±3.4*^{a}
1000	8.2±0.8	8.9±1.3^{b}	3.1±0.2	4.6±0.6*^{b}	248.1±40.1	202.0±28.9	38.5±1.6	49.7±2.3*^{b}

The symbol *indicates significant difference before and after treatment within each group at $p<0.05$, The same alphabet indicates significant difference between groups at $p<0.05$

Table 3: Liver and kidney weights in the subacute toxicity study

	Treatment groups			
Organs	Control (0 mg kg^{-1})	200 mg kg^{-1}	500 mg kg^{-1}	1000 mg kg^{-1}
Liver (g)	1.52±0.07	1.31±0.21	1.11±0.04	1.33±0.12
Kidneys (g)	0.32±0.01^{ab}	0.26±0.02^{a}	0.25±0.01^{b}	0.29±0.02

The symbol * indicates significant difference before and after treatment within each group at $p<0.05$. The same alphabet indicates significant difference between groups at $p<0.05$

Table 4: Liver and kidney weights in the subchronic toxicity study

	Treatment groups			
Organs	Control 0 mg kg^{-1}	200 mg kg^{-1}	500 mg kg^{-1}	1000 mg kg^{-1}
Liver (g)	1.51±0.1	1.44±0.11	1.64±0.22	1.19±0.05
Kidneys (g)	0.33±0.03^{a}	0.30±0.02	0.27±0.02	0.24±0.01^{a}

The symbol * indicates significant difference before and after treatment within each group at $p<0.05$. The same alphabet indicates significant difference between groups at $p<0.05$

Serum aspartate aminotransferase and creatinine: No changes were observed in serum AST levels in the Subacute and Subchronic Toxicity Studies (Table 1, 2). Serum Creatinine was higher in the 500 and 1000 mg kg^{-1} group compared to Control in the Subchronic Toxicity Studies (Table 2), while no changes were observed in the Subacute Toxicity Study (Table 1).

Liver and kidney weights: No significant differences in liver weights between groups were observed in both the Subacute and Subchronic Toxicity Studies (Table 3 and 4). However, kidney weights were lower in the 200 and 500 mg kg^{-1} group compared to Control for the Subacute Toxicity Study (Table 3) and lower in the 1000 mg kg^{-1} group compared to Control for the Subchronic Toxicity Study (Table 4).

DISCUSSION

Body weight of mice in the Subacute Toxicity Study did not change throughout the 2-week study period for all the groups including the Control group. This indicates that the doses of palm tocotrienols used did not impair overall growth of the animal in the short-term. However, in the Subchronic Toxicity Study, body weights of the animals given 500 and 1000 mg kg^{-1} began to decline from the fourth week onwards. These findings are consistent with the mortality data, whereby no deaths were recorded in the Subacute Toxicity Study, but some deaths were seen in

the Subchronic Toxicity Study. The first death occurred at 4 weeks of treatment in the group given the highest dose, i.e., 1000 mg kg^{-1}. There are some differences in these results compared to that found by others. In Oo *et al.* (1992), gave higher doses of palm vitamin E mixture to mice and rats, i.e., between 250 to 2500 mg kg^{-1} for 30 days but did not record any mortality. Nakamura *et al.* (2001) administered 0.19, 0.75 and 3% tocotrienols in a powdered diet preparation and did not observe any deaths in his laboratory rats. There could be various reasons to account for the difference in these studies, some of them being the different species and strains of the animals used, different duration of treatment as well as differences in the tocotrienol preparations.

In this study, both the Bleeding Time and Clotting Time were impaired in the Subacute and Subchronic Toxicity Studies beginning from the 500 mg kg^{-1} dose onwards. Past research on toxicity of vitamin E was mainly done on tocopherols. Large doses of vitamin E, namely alpha-tocopherol, have been associated with bleeding in animal studies, which were reversed by vitamin K supplementation (Wheldon *et al.*, 1983). The upper tolerable limit for vitamin E, i.e., 1000 mg day^{-1} for humans, set by the American Food and Nutrition Board was based on studies in rats which showed that the adverse effect caused by excess vitamin E was bleeding tendency (Monsen, 2000, Traber, 2008. Thus from this study it appeared that tocotrienols have the same tendency as tocopherols to cause bleeding most probably due to interaction with vitamin K. There is a potential for this property of vitamin E to be utilised as an anticoagulant. Since patients with coronary heart disease typically exhibit increased levels of oxidative stress together with decreased antioxidant enzyme activities and increased platelet aggregation, it was suggested that supplementation of moderate levels of vitamin E and a-lipoic acid could provide both antioxidant and anti-coagulant effects that could benefit these patients (Marsh and Coombes, 2009). Wang *et al.* (2009) also found that pre-treatment with vitamin E reduced the risk of thrombosis associated with hypoxic exposure in healthy men, suggesting the potential benefit of vitamin E in preventing stroke. Thus the anticoagulant effects of tocotrienols need to be further explored.

No hepatotoxicity due to tocotrienols was seen at the doses used in this study as evidenced by normal serum aspartate transaminase levels and normal liver weights. However, some degree of renal impairment was evidenced by the increase in serum creatinine in the 500 and 1000 mg kg^{-1} dose in the Subchronic Toxicity Study. This coincides with the reduction in kidney weights seen in the 200 and 500 mg kg^{-1} doses in the Subacute Toxicity Study; as well as in the 1000 mg kg^{-1} dose in the Subchronic Toxicity Study. No animal or human studies have so far shown any significant renal toxicity of vitamin E, either with the tocopherols or the tocotrienols. However, this study suggests some form of renal impairment, especially at the higher doses of vitamin E used. This finding needs further elucidation and confirmation.

The American Food and Nutrition Board recommends a daily intake of 15 mg vitamin E for male and female adults from 19 years of age onwards and an upper tolerable limit of 1000 mg day^{-1} for the same age group (Monsen, 2000). The non-toxic dose of 200 and the toxic dose of 500 mg/kg/day used in mice in this study can be extrapolated to 1,400 and 3,500 mg/day for humans which are far higher than the upper tolerable limit of 1,000 mg/kg/day. Furthermore, the effective dose of palm vitamin E extract to prevent and treat osteoporosis that we have discovered in our earlier animal studies was 60 mg kg^{-1} (Ahmad *et al.*, 2005; Hermizi *et al* 2009; Ima-Nirwana and Suhaniza 2004; Nazrun *et al.*, 2005, 2008), which can be extrapolated to 420 mg/day in humans. Furthermore our effective dose of 60 mg/kg/day for the rats is much lower than the toxic dose of 500 mg kg^{-1} in mice found in this study. Thus we conclude from this study that doses of palm tocotrienols well above the upper tolerable limit of 1,000 mg day^{-1} may cause bleeding

tendency and renal impairment. However, doses at and below the upper tolerable limit are free of any adverse effects. Therefore, the dose of 60 mg kg^{-1} in rats that was effective in preventing and treating osteoporosis is safe when extrapolated to humans.

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